Degradation of carbofuran by an enrichment culture developed from carbofuran-treated *Azolla* plot

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Abstract: Standing water from carbofuran-treated *Azolla* plots showing accelerated degradation was further enriched by five repeated transfers to carbofuran-supplemented mineral salts medium. This enrichment culture developed from standing water of carbofuran-treated *Azolla* plot can utilise carbofuran as sole source of carbon and nitrogen. The enrichment culture was able to hydrolyse nearly 100% of [*ring*-^{14}C]carbofuran to carbofuran phenol in five days, which accumulated in the medium, while the carbamate side-chain in [*carbonyl*-^{14}C]carbofuran was readily mineralized to [^{14}C]carbon dioxide. Enrichment culture was able to degrade carbofuran up to 1000 μg ml^{-1} levels in mineral salts medium with ease.

Keywords: enrichment culture; *Azolla*; carbofuran; accelerated degradation; high concentrations

1 INTRODUCTION

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is an extensively used broad-spectrum insecticide and nematicide. There have been reports that carbofuran fails to control insects after it has been used continuously for several years.[1–4] The reduction in efficacy of carbofuran could result from the fact that target insects may develop resistance, or soil micro-organisms that repeatedly encounter synthetic xenobiotics may develop new capabilities to degrade these. It is well established that soil micro-organisms play an important role in the rapid degradation of carbofuran,[1,5–10] if used continuously for several years. *Azolla*, a water fern harbouring a nitrogen-fixing blue-green alga, *Anabaena azollae*, is used as a biofertillzer in rice cultivation.[11] *Azolla* is maintained throughout the year at the experimental farm of the Central Rice Research Institute, Cuttack, India. Carbofuran is used to protect it from certain insect pests at the rate of 1 to 1.5 kg AI ha^{-1} and five to ten applications are given in a year, depending upon disease incidence. As these plots are regularly treated with carbofuran, standing water from some *Azolla* plots has developed the ability to degrade carbofuran.[12] When carbofuran was mixed with water from *Azolla* plots retreated with carbofuran, only three of the six plots exhibited accelerated degradation of carbofuran. Thus, the insecticide completely disappeared in 10 days when mixed with water from three of the six plots. During the same period, almost 70% of the added insecticide was recovered from water of the three remaining treated plots or from the water of a plot not previously treated with carbofuran. Therefore, an enrichment culture prepared by further additions of carbofuran to standing water of one of the three *Azolla* plots showing accelerated degradation was used to study degradation of carbofuran in mineral salts medium, and the results are discussed here.

2 MATERIALS AND METHODS

2.1 Insecticide and metabolites

Analytical grade carbofuran (99.5%), [*U-ring*-^{14}C]carbofuran (specific activity 39.4 mCi mmol^{-1}, 50% purity), [carbonyl-^{14}C]-carbofuran (specific activity 13.3 mCi mmol^{-1}, 98% purity), carbofuran phenol, 3-ketocarbofuran and 3-hydroxycarbofuran were obtained from FMC Corporation Middleport, New York.

2.2 Preparation of carbofuran-degrading enrichment culture

Azolla plot water, pH 7.1 (5 ml), which had been treated regularly with carbofuran (five to ten applications in a year) to protect it from insect pests and which exhibited enhanced degradation of carbofuran, was mixed with 5 ml of mineral salts medium [$MgSO_4$. $7H_2O$, (0.2); K_2HPO_4, (0.1); $FeSO_4$. $7H_20$, (0.001); $CaSO_4$, (0.04) g litre^{-1} distilled water) pH, 6.2], supplemented with 20 μg ml^{-1} of carbofuran. Carbo-

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furan was added to the flasks in 1 ml of acetone and, after the evaporation of the acetone, medium was added to the flasks. After 10 days, 5 ml of this carbofuran-degrading culture was further mixed with 5 ml of fresh mineral salts medium containing carbofuran. The process was repeated thrice more. After the fifth transfer, its ability to degrade [^{14}C]carbofuran in mineral salts medium was tested

2.3 Degradation of [^{14}C]carbofuran by enrichment culture

[*U-ring*-^{14}C]carbofuran (1.7×10^5 DPM) or [*carbonyl*-^{14}C]carbofuran (2.2×10^5 DPM) in 1 ml of acetone was added aseptically to presterillzed 100-ml Erlenmeyer flasks. After evaporation of acetone at room temperature, 20-ml portions of sterile mineral salts medium were dispensed into these flasks. After equilibration for 24 h, the medium in duplicate flasks was inoculated with 0.2 ml of enrichment culture. Uninoculated mineral salts medium in duplicate flasks with carbofuran served as a control. Flasks were closed with a rubber bung fitted with an inlet and an outlet, which were closed with a pinchcock, and flasks were incubated at room temperature [27(±2) °C]. At regular intervals, the inlet was connected to an air generator through a trap containing potassium hydroxide solution (2 M; 25 ml) to remove [^{14}C]carbon dioxide, if any, from the air. The [^{14}C]carbon dioxide that evolved from [^{14}C]carbofuran from each of the duplicate flasks containing inoculated and uninoculated medium was purged, after the pinchcock was released, into 5 ml of scintillation cocktail (repurged with nitrogen) containing pseudocumene (R J Harvey Instrument Corporation, 123 Patterson Street, New Jersey, USA). The contents in each reaction flask were shaken with chloroform + diethyl ether for extraction of residues and subsequent assay by liquid scintillation after separation by thin-layer chromatography.

2.4 Degradation of carbofuran by enrichment culture as a function of its concentration

Analytical grade carbofuran in 1 ml of acetone was added aseptically to presterilized 100-ml Erlenmeyer flasks to obtain final concentrations of 20, 50, 100, 500 and 1000 μg ml^{-1}. After evaporation of acetone at room temperature, 20 ml portions of sterile mineral salts medium were added to these flasks. After equilibration for 24 h, flasks were inoculated with 0.2 ml of enrichment culture obtained from an *Azolla* plot. Uninoculated medium served as a control. The flasks were incubated at 35(±1) °C. At regular intervals, duplicate flasks were removed for extraction and residue analysis by colorimetry.

2.5 Extraction and residue analysis

Residues from the medium were extracted with chloroform + diethyl ether (1 + 1 by volume; 3×30 ml). The extracts were pooled and evaporated to dryness or 0.5 ml as certain metabolites may be volatilized from glass surface during evaporation of the solvent to dryness. Residues were redissolved in methanol and were separated by thin-layer chromatography as described by Ramanand *et al.*[13] The standards were located by spraying successively with sodium hydroxide in methanol (2 M) and a solution of *p*-nitrobenzene diazonium fluoroborate (5 mg) in methanol + diethyl ether (1 + 1 by volume; 50 ml) as described by Archer.[14] Silica gel areas corresponding with the standards were scraped into tubes for colorimetric analysis or into 5 ml of scintillation cocktail to assay ^{14}C activity.

For colorimetric analysis of carbofuran, residues in silica gel were treated with sodium nitrite (3 g litre^{-1}; 1.25 ml), sulfanilic acid (1.25 ml) in hydrochloric acid (1 M), and sodium hydroxide (4 M; 2.5 ml), and incubated on water bath (40–50 °C) for 20 min. Silica gel was removed by centrifugation (2800 *g* for 30 min) and the supernatant was made up to 10 ml with distilled water prior to colorimetric analysis[15] at 490 nm. Recovery of carbofuran by this method ranged from 85 to 90%.

In isotope studies, the silica gel areas corresponding to authentic carbofuran and metabolites were scraped into 5 ml of scintillation cocktail (FSA Laboratory Suppliers, Loughborough, Leics, UK) and radioactivity assayed in a Rackbeta Model 1209 liquid scintillation spectrometer (LKB, Wallac, Finland), programmed for colour and chemical quenching correction. DPM conversion with background quenching correction was done by Facit B 1100 printer interfaced with the liquid scintillation spectrometer. [^{14}C] Carbon dioxide absorbed in scintillation cocktail was assayed directly by liquid scintillation.

The thin-layer chromatograms of the solvent extracts of the uninoculated and inoculated mineral salts medium containing [^{14}C]carbofuran and its degradation products were exposed to Kodak X-ray screen film for 30 days in a Siemen's metal cassette and the film was developed with X-ray developer.

3 RESULTS AND DISCUSSION

3.1 Degradation of [^{14}C]carbofuran by enrichment culture

The enrichment culture obtained from the standing water of carbofuran-treated *Azolla* plots showing enhanced degradation of carbofuran, after further repeated transfer of *Azolla* plot water to carbofuran-containing mineral salts medium, demonstrated an exceptionally high ability to degrade carbofuran in mineral salts medium. Standing water of carbofuran-retreated *Azolla* plot completely degraded carbofuran in 10 days.[12] The enrichment culture prepared from standing water after five further transfers to carbofuran-supplemented medium degraded it almost completely in five days (Table 1). Total radioactivity extracted and eluted in methanol from inoculated mineral salts medium containing [*ring*-^{14}C]carbofuran decreased with incubation and reached low levels within five days of incubation. After five days, the

Table 1. Degradation of [U-*ring*-^{14}C]-carbofuran in mineral salts medium by the enrichment culture obtained from carbofuran-treated *Azolla* plots (involving complete evaporation of chloroform-diethyl ether extract)

		Radioactivity[a] recovered (%) (±S.D)							
Incubation (days)	Treatment	Aqueous phase	Methanol extract	Carbofuran	Carbofuran phenol	3-Keto-carbofuran	3-Hydroxy-carbofuran	CO_2	Total recovery
0	Uninocluated Inoculated	1.1 (±0.1)	91.9 (±1.1)	89.2 (±1.0)	0.6 (±0.1)	–	–	–	93.0
2	Uninoculated	1.3 (±0.2)	87.9 (±2.0)	82.0 (±0.9)	0.4 (±0.1)	1.6 (±0.0)	1.1 (±0.1)	0.2 (±0.0)	89.4
	Inoculated	1.2 (±0.1)	54.1 (±1.4)	38.4 (±2.3)	1.1 (±0.2)	0.8 (±0.1)	1.2 (±0.4)	0.3 (±0.1)	55.5
5	Uninoculated	2.5 (±0.3)	84.5 (±0.3)	79.8 (±1.1)	0.7 (±0.3)	1.5 (±0.1)	1.0 (±0.3)	0.3 (±0.1)	87.3
	Inoculated	2.5 (±0.2)	26.6 (±1.0)	4.8 (±0.6)	8.3 (±0.8)	1.0 (±0.2)	0.6 (±0.2)	0.7 (±0.2)	29.8

[a] The mineral salts medium was supplemented with labelled carbofuran (1.7×10^5 dpm $(20\,ml)^{-1}$).

Table 2. Degradation of [U-*ring*-^{14}C]-carbofuran in mineral salts medium by the enrichment culture obtained from carbofuran-treated *Azolla* plots (involving partial evaporation of chloroform-diethyl ether extract)

		Radioactivity[a] recovered (%) (±S.D)							
Incubation (days)	Treatment	Aqueous phase	Methanol extract	Carbofuran	Carbofuran phenol	3-Keto-carbofuran	3-Hydroxy-carbofuran	CO_2	Total recovery
0	Uninoculated Inoculated	1.0 (±0.2)	92.2 (±4.0)	88.6 (±4.8)	0.5 (±0.0)	–	–	–	93.2
2	Uninoculated	1.3 (±0.2)	87.8 (±3.4)	82.6 (±3.9)	0.4 (±0.1)	1.4 (±1.0)	1.0 (±0.0)	0.2 (±0.1)	89.1
	Inoculated	1.2 (±0.6)	74.1 (±2.3)	38.4 (±3.0)	21.1 (±0.3)	0.8 (±0.2)	1.2 (±0.2)	0.4 (±0.0)	75.7
5	Uninoculated	2.5 (±0.7)	83.1 (±4.8)	78.8 (±2.9)	0.7 (±0.2)	1.6 (±0.3)	0.8 (±0.1)	0.4 (±0.1)	86.0
	Inoculated	2.4 (±0.4)	72.6 (±3.4)	4.7 (±0.2)	51.3 (±1.2)	0.9 (±0.1)	0.9 (±0.3)	0.6 (±0.2)	75.6

[a] The mineral salts medium was supplemented with labelled carbofuran (1.7×10^5 dpm $(20\,ml)^{-1}$).

quantity of [*ring*-^{14}C]carbofuran fell to 4.8% of the initially added radioactivity in the inoculated medium, while there was only slight loss in the [^{14}C]carbofuran, extractable by organic solvent, in the uninoculated medium. Carbofuran phenol, the major hydrolysis product from ring-labelled carbofuran, was detected, but not in stoichiometric amounts. After five days, 8.3% of the ^{14}C in [*ring*-^{14}C]carbofuran accumulated as carbofuran phenol. Formation of carbofuran phenol, though not in a proportionate amount, indicates hydrolysis during the degradation of carbofuran by the enrichment culture. 3-Ketocarbofuran and 3-hydroxycarbofuran were detected in trace quantities (1–3% of initially added *ring*-^{14}C). About 0.2–0.7% of the ^{14}C in the [*ring*-^{14}C]carbofuran was removed as [^{14}C]carbon dioxide from the inoculated and uninoculated medium in five days, indicating that the ring is cleaved to only a small extent during degradation. Interestingly, radioactivity remaining in the water phase after extraction was negligible.

Most of the radioactivity added to the uninoculated medium was accounted for, but this was not so for the inoculated medium. This decrease in radioactivity from inoculated medium could not be due to volatilization or chemical losses of carbofuran as only 10% of the radioactivity was lost from the uninoculated medium during the same period. Also, during incubation, the pH of the medium was always below 7. This suggests the formation of metabolite(s) in inoculated medium which is either volatile (other than carbon dioxide) or lost during the extraction and evaporation of the organic solvent mixture.

In a follow-up study, the above experiment with [*ring*-^{14}C]carbofuran was repeated, but the chloroform + diethyl ether extract was evaporated to about 0.5 ml and not to complete dryness. Interestingly, 72% of the ^{14}C in [*ring*-^{14}C]carbofuran was found in the organic solvent extract from the inoculated medium when the chloroform + diethyl ether extract was not evaporated to dryness (Table 2). Analysis of ^{14}C after thin-layer chromatographic separation of residues showed that 51% of the ^{14}C in [*ring*-^{14}C]carbofuran in the inoculated medium was accounted for as carbofuran phenol. Autoradiography of the partially evaporated organic extract showed the presence of only one prominent metabolite which had the same R_f as authentic carbofuran phenol. Evidently, the metabolite formed from carbofuran was carbofuran phenol which was lost from the glass surface during evaporation of the chloroform-diethyl ether extract to dryness. Similar results were obtained by Venkateswarlu *et al*,[15] which showed that an enrichment culture formed after repeated additions of carbofuran to flooded soil could degrade 60–70% of carbofuran in 40 days and carbofuran phenol was recovered as the major metabolite. However, Ramanand *et al*[13] reported that

Table 3. Degradation of [*carbonyl*-^{14}C]carbofuran in mineral salts medium by the enrichment culture obtained from carbofuran-treated *Azolla* plots

		Radioactivity[a] *recovered (%) (±SD)*				
Incubation (days)	*Treatment*	*Aqueous phase*	*Methanol extract*	*Carbofuran*	*CO_2*	*Total recovery*
0	Uninoculated	2.6 (±0.6)	95.3 (±1.3)	89.3 (±2.0)	–	97.9
	Inoculated					
2	Uninoculated	4.7 (±0.8)	86.9 (±2.0)	84.7 (±1.8)	5.3 (±1.0)	96.9
	Inoculated	2.6 (±0.7)	3.7 (±0.3)	1.5 (±0.2)	74.2 (±3.0)	80.5
5	Uninoculated	3.8 (±0.0)	81.1 (±3.2)	80.0 (±2.8)	5.5 (±0.3)	90.4
	Inoculated	2.3 (±0.2)	2.0 (±0.4)	0.8 (±0.4)	75.5 (±2.5)	79.8

[a] The mineral salts medium was supplemented with labelled carbofuran (2.2×10^5 dpm (20 ml)$^{-1}$).

an enrichment culture obtained after repeated additions of carbofuran to an alluvial soil at 35 °C degraded [*ring*-^{14}C]carbofuran to negligible levels in five days and carbofuran phenol accumulated as a transitory metabolite, which was further mineralized to [^{14}C]carbon dioxide. Recently, Trabue *et al*[16] and Talebi and Walker[9] have shown that carbofuran is degraded by hydrolysis of the ester linkage in soils which have been previously treated with carbofuran.

In another study, a mineral salts medium containing [*carbonyl*-^{14}C]carbofuran was inoculated with enrichment culture. The total radioactivity in the organic solvent extract of inoculated medium decreased to 3.7% of the original level in two days compared to a recovery of 86.9% from the uninoculated medium (Table 3). Analysis of the methanol extract after thin-layer chromatographic separation showed that only 1.5% of activity was accounted for as carbofuran in inoculated medium while 84.7% of the ^{14}C in uninoculated medium was recovered as carbofuran. It is interesting to note that 72–75% of the ^{14}C in [*carbonyl*-^{14}C]carbofuran was recovered as [^{14}C]carbon dioxide from the inoculated medium. Evidently, the enrichment culture used in the study was capable of releasing [^{14}C]carbon dioxide from carbonyl-labelled carbofuran with great ease. According to an earlier report from the same laboratory,[13] a substantial portion of the radioactivity from [*carbonyl*-^{14}C]carbofuran during its degradation by enrichment culture was not accounted for, as no [^{14}C]carbon dioxide was evolved. Kuhr and Dorough[17] reported that degradation of carbofuran proceeded by primary hydrolysis of the carbamate linkage leading to the formation of carbofuran phenol and methyl isocyanate. Methyl isocyanate, being highly volatile, may escape from the system before it is acted upon by micro-organisms. However, in the present study, most of the carbonyl-^{14}C is accounted for as [^{14}C]carbon dioxide, indicating that the micro-organisms present in the enrichment culture are able to mineralize the carbamate side chain to [^{14}C]carbon dioxide and methylamine.

3.2 Degradation of carbofuran as function of its concentration

The enrichment culture was able to degrade carbofuran at all selected concentrations of 20, 50, 100, 500 and 1000 μg ml^{-1} of medium with great ease (Table 4).

Table 4. Degradation of carbofuran in mineral salts medium at 35 °C by the enrichment culture (as influenced by different concentration levels) obtained from carbofuran-treated *Azolla* plots

	Carbofuran recovered[a] *(μg ml^{-1}) (±SD)*									
	20		*50*		*100*		*500*		*1000*	
Incubation (days)	*U*[b]	*I*[c]	*U*	*I*	*U*	*I*	*U*	*I*	*U*	*I*
0	18.7 (±0.3)	19.5 (±0.5)	49.9 (±0.9)	46.0 (±1.0)	89.0 (±1.5)	89.0 (±1.0)	465.0 (±5.0)	470.0 (±5.0)	880.0 (±10.0)	875.0 (±5.0)
2	18.9 (±0.1)	0	45.0 (±2.0)	34.5 (±1.5)	87.5 (±0.5)	73.5 (±1.5)	ND[d]	ND	ND	ND
5	NE	NE	41.0 (±1.0)	0	82.0 (±1.0)	15.0 (±1.0)	440.0 (±10.0)	222.0 (±8.0)	845.0 (±5.0)	760.0 (±10.0)
10	NE	NE	NE	NE	74.0 (±1.0)	0	405.0 (±5.0)	84.5 (±2.5)	770.0 (±13.0)	680.0 (±10.0)
15	NE	NE	NE	NE	NE	NE	330.0 (±15.0)	5.5 (±3.7)	605.0 (±13.8)	230.0 (±15.0)

[a] The mineral salts medium was supplemented with 20, 50, 100, 500 and 1000 μg of carbofuran ml^{-1} medium.
[b] Uninoculated.
[c] Inoculated.
[d] ND: not determined.

Carbofuran at concentrations of 20, 50 and 100 $\mu g\,ml^{-1}$ medium reached undetectable levels in 2, 5 and 10 days, respectively. Nearly 98% of initially added carbofuran was degraded in 15 days in medium containing carbofuran at 500 $\mu g\,ml^{-1}$ medium. During the corresponding period, about 73% of carbofuran was degraded at the highest concentration of 1000 $\mu g\,ml^{-1}$ medium. Even when carbofuran was added to the medium at high concentrations of 500 and 1000 $\mu g\,ml^{-1}$ medium, its concentration in solution would be around its solubility level of *c* 350 $\mu g\,ml^{-1}$. Carbofuran added at the 1000 $\mu g\,ml^{-1}$ level was also degraded, but after an initial lag, indicating that carbofuran is not toxic at this level. There is about 25% loss of carbofuran in 10 days from uninoculated medium also, which is attributed to volatilization loss of carbofuran at the high temperature of 35 °C. Earlier, Read[18] has shown that carbofuran added at concentrations from 10 to 5000 $\mu g\,g^{-1}$ of soil was degraded to levels not detected by the bioassay method in less than one day at 10 $\mu g\,g^{-1}$ soil and 90 days at 5000 $\mu g\,g^{-1}$ soil. Concentrations from 10 to 500 $\mu g\,g^{-1}$ soil were degraded quickly, while higher concentrations (1000–5000 $\mu g\,g^{-1}$ soil) were degraded slowly.

The results indicate that repeated exposure/application of carbofuran to standing water from *Azolla* plots for long periods results in the evolution of micro-organisms capable of degrading the insecticide rapidly and extensively. Isolation and characterization of micro-organisms from such enrichment cultures in which carbofuran is rapidly inactivated would be useful. Also, since they can withstand the high concentrations of carbofuran and can degrade it easily, such micro-organisms could be used to decontaminate pesticide-polluted sites.

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